

Oligomers Derived from Natural PHA

4

Bioactive and Functional Oligomers Derived from Natural PHA and Their Synthetic Analogs

Anabel Itohowo Ekere, Iza Radecka, Piotr Kurcok, Grazyna Adamus, Tomasz Konieczny, Magdalena Zięba, Paweł Chaber, Fideline Tchuenbou-Magaia, and Marek Kowalczyk

4.1 Introduction

Polyhydroxyalkanoates (PHA) are biopolyesters with good biodegradability and biocompatibility with broad application potential. The composition of monomeric building blocks, microstructures, and supra-macromolecular architecture determine the chemo-mechanical properties of PHA, and thus their suitability for defined technological applications [1, 2]. PHA were first discovered in the cytosols of certain microorganisms as high molar mass polymers stored in the form of granules [1]. These high molar mass PHA have more than 1 000 3-hydroxyacid residue units (approximately 50–5 000 kDa) and are generally referred to as storage PHA (sPHA) [3]. In certain prokaryotic bacteria, PHA are synthesized intracellularly in the form of inclusion bodies in their cytosols with their main function being for carbon and energy [4]. Much later, a low molar mass form of PHA referred to as PHA oligomers was discovered and reported by Reusch and Sadoff [5]. Consequently, PHA have now been classified into high molar mass PHA and low molar mass PHA (PHA oligomers), based on their molar mass.

PHA oligomers (oligo-PHA) are low molar mass PHA consisting of a small number of 3-hydroxyacids repeat units, usually three or more 3-hydroxyacids residue units, but not more than 200 residue units [6]. They are built from the same monomer units as sPHA, but possess a much shorter chain. While sPHA are primarily formed in many prokaryotes (Eubacteria and Archaea), PHA oligomers are components of all prokaryotes and eukaryotes and are thus thought to be present in all living organisms [3].

Over the last few years, storage PHA have been extensively studied and considered a more attractive biomaterial in research with high future impact due to their desirable properties and extensive applications. Nevertheless, the insufficient yields and poor mechanical properties of PHA limit its widespread commercialization. This is partly due to their hydrophobic nature (a long-chain fatty hydroxyl acid molecule) that results in fewer functional groups [7]. As a result, their applications in other advanced areas of importance are limited. Thus, to expand their widespread commercialization and improve their potential applications, PHA would need to have appropriate hydrolytic stability and enhanced chemical functionalities. This would improve their mechanical properties, amphiphilic character, and surface structure to meet the requirements of their tailored application [7, 8]. To achieve this, PHA oligomers containing reactive functional end groups with controlled -molar mass can be used as building blocks of new block biopolymers with enhanced properties [9]. Furthermore, the high biocompatibility, bioactive function, and block copolymerisation of PHA oligomers with other polymers have been discovered to exhibit desirable properties for valued-added biomaterial applications, especially in medicine for therapeutic use, cosmetology, and agrichemistry [2, 10, 11]. Moreover, several researchers have also identified and confirmed the various range of PHA oligomers with high potential for being a good source of reactive oligoesters [12, 13, 14, 15, 16]. Thus, with these developments, it is expected that there would be an amplified interest in the functionalization of PHA biomaterials for other novel applications.

The physical properties of PHA oligomers are chiefly dependent on the length of the chain [17]. Aside from being biodegradable, PHA oligomers also exhibit excellent biocompatibility as confirmed by their lack of toxicity. This is well demonstrated by the natural presence of relatively large amounts of low-molar mass poly(3-hydroxybutyrate) (PHB) and other related oligomers in the bloodstream of humans [18]. In addition, PHA oligomers are components of all living organisms including eukaryotic cells and mammals where they are widely

distributed in various intracellular fluids and fractions of these cells. In most cases, a short-chain form of low molecular mass (oligo-PHA) PHA is complexed to other cellular macromolecules such as proteins and inorganic polyphosphates and thus referred to as complexed PHA (cPHA).

4.2 Oligomers Derived from Natural Storage

4.2.1 Oligo-PHB

Oligo-PHB was first discovered by [5] in the cytoplasmic membrane of *Azotobacter vinelandii* and *Bacillus subtilis*, comprising about 100–200 monomeric units. Oligo-PHB is known to often form complexes with calcium ions and polyphosphate (polyP) and is suggested to be responsible for the uptake of DNA during transformation [3]. Oligo-PHB is also part of low-density lipoproteins in human plasma and thus is suggested to be involved in arteriosclerosis [19].

4.2.2 Complexed PHB

cPHB was first discovered in *Escherichia coli*, but it has ever since then been found to be a ubiquitous constituent of both prokaryotic and eukaryotic cells. They usually consist of a low number of 3HB units (<~30) [3, 20] and are widely distributed in numerous cell fractions mostly in complex with other bio-macromolecules including inorganic polyPs and proteins [21]. They are usually water-soluble and chloroform-insoluble as long as the protein molecule to which it is attached to is water-soluble [3]. The majority of cPHB is complexed covalently with proteins, while a small fraction of them are non-covalently complexed with calcium polyP [22]. There is evidence from several reporters that these polyP complexes play a major role in the acquisition of competence in *Escherichia coli*, where they form ion channels in their plasma membranes [23, 24]. In addition, the covalent modification of proteins by cPHB, referred to as PHBylation, could have an important effect on the physiological properties of proteins. A significant amount of cPHB in *E. coli* is dependent on

the presence of a novel type of PHB synthase called YdcS, different from sPHB synthase [25].

4.2.3 Synthesis of Natural PHA Oligomers

Oligomeric PHA could be synthesized either through intracellular degradation of PHA yielding natural oligomers, extracellular degradation or chemical modifications to yield synthetic oligomers. These pathways have been identified and confirmed by several researchers [26, 27, 28, 29]. The extracellular degradation and chemical modifications for oligomer synthesis have been reported by many studies [9, 30, 31, 32], but only a few studies have been published on the intracellular degradation of PHA [33, 34].

4.2.4 Intracellular Degradation of Storage PHA

Intracellular degradation is the natural means by which PHA oligomers are being synthesized; it is, however, the less common route by which oligomers are synthesized, as gathered from the literature. This is because of the minute quantity of oligomeric PHA in cells, and thus the cellular mechanism behind the intracellular synthesis of PHA oligomers is yet to be fully understood.

So far, PHB has been extensively used to study the intracellular metabolism of PHA.

Intracellular sPHB when accumulated with some intact surface layer and some PHB binding proteins in bacteria cells are referred to as native PHB (nPHB) or amorphous PHB (aPHB) [35]. If, however, these PHB-producing cells die (e.g. after cell lysis or solvent extraction) with or without a damaged surface layer, they are released into the environment, denature, and turn out to be more or less crystalline (semicrystalline/paracrystalline state) [36].

Although the extracellular degradation of denatured crystalline, PHB (dPHB), has been amplified in numerous bacteria, only a little is known about the intracellular degradation of native PHB [36].

Based on reported *in vivo* studies, sPHBs are usually degraded intracellularly by PHB depolymerase (PhaZ1) to obtain natural PHA oligomers [34]. PhaZ1 has been found to occur only as a form bound to PHA granules in cells, with various 3-hydroxybutyrate (3HB) oligomers being its major hydrolytic products from the enzymatic degradation of amorphous PHB [35]. This *in vivo* degradation process as described for *Ralstonia eutropha* (today: *Cupriavidus necator*) starts in the chains of amorphous PHB molecules. In this process, several links are created in the chain by intracellular PHB depolymerase (iPHB-PhaZ1) bound to exclusively inclusion bodies [33, 37]. Consequently, medium-sized 3HB-oligomers (that still bind to the granules) are produced mostly due to their hydrophobicity. In addition to this, some loosened 3HB ends of PHB chains protruding from the granules are produced, as well as small amounts of short-chain 3HB-oligomers (3–5 units) which diffuse from the granules [33, 34]. However, as reported by several researchers, this is only applicable to native or amorphous PHB granules but not crystalline PHB granules [27, 38].

Asides *R. eutropha*, there have been other reports on the intracellular degradation of sPHB in other bacteria such as *Escherichia coli* [35, 39, 40]. In most of these reported studies, PhaZ1 of *Ralstonai eurtropha* H16 was cloned and just a few novel intracellular PhaZ genes were identified and characterized [27, 40, 41]. In a report by Saegusa and his colleagues [40], an intracellular poly[D(-)-3-hydroxybutyrate] depolymerase gene (PhaZ) was cloned from *R. eutropha* and expressed in *E. coli*. The crude extract of *E. coli* containing PhaZ gene digested amorphous PHB granules and released majorly oligomeric D(-)-3-hydroxybutyrate with the addition of some monomers [40]. Furthermore, it was reported in recent studies that an increase in PhaZ activity during culturing of *A. vinelandii* led to a decrease in the average molecular weight of sPHB and an increase in the fraction of low molecular weight PHB [27, 38, 42]. Besides these studies, no other study to the best of our knowledge has fully elucidated the metabolism behind the intracellular accumulation of PHA oligomers.

4.2.5 Extracellular Degradation of Storage PHA

Storage PHA that were synthesized by humans or released from PHA-accumulating microorganisms can be easily cleaved to by extracellular PHB depolymerases and many researchers have described numerous examples of extracellular PHB depolymerases over the last few decades [43, 44, 45, 46]. A good number of microbes, particularly bacteria and fungi in soil, sludge, and seawater have been reported to excrete extracellular PHA-degrading enzymes able to hydrolyze sPHA into water-soluble oligomers [29]. Subsequently, these organisms make use of the resulting products as nutrients within their cells. In one of these studies carried out by Sugiyama *et al.* [31], PHB depolymerases (PHBDPs) were purified from two different bacteria (*Ralstonia pickettii* T1 and *Acidovorax* sp. SA1) to investigate their degradation products, kinetic properties, and substrate specificity. It was discovered that for *Ralstonia pickettii* T1, an extracellular PHB depolymerases enzyme degrades extracellular PHB to numerous sized 3HB-oligomers, and for *Acidovorax* sp. SA1, an extracellular PHBDP hydrolyzes extracellular PHB to small 3HB-oligomers (dimer and trimer) [31]. In another study by Gebauer and Jendrossek [36], protease-treated PHA granules were degraded with extracellular PHA depolymerases isolated from *P. lemoignei* to obtain 3HB and 3HV oligomers. In addition, Utsunomia and his colleagues [30] also recently reported on the production of D-lactate-3HB oligomers (D-LAOs) by engineered *Escherichia coli* expressing a D-specific lactate polymerizing enzyme. The extracted oligomers in this study contained 45 mol-% LA and 55 mol-% 3HB, suggesting that the obtained D-LAOs-DEG are random copolymers of LA and 3HB. The secreted D-LAOs also possessed two hydroxyl end group that can be used as building blocks for the production of numerous LA-based polymers including LA-based poly(ester-urethane) (PEU) [30].

4.2.6 Extraction of PHA Oligomers

The detailed extraction of PHA oligomers from cells have been previously described by Reusch and Sadoff [47], which has also been modified by Seebach *et al.* and Suzuki *et al.* [48, 49].

Due to the low concentrations of cPHB in certain solvents comprising of either lipids only, or lipids with proteins, its physical properties are usually modified by these solvents. Thus, the solubility of cPHB is majorly dependent on the properties of its complexant, where cPHB may be found in the residue part of its complexant, in any of the wash solutions or in the aqueous hypochlorite supernatant [19]. Even when cPHB is isolated from samples with chloroalkanes, cPHB would most often fail to dissolve in boiling chloroform. Consequently, designing a general procedure for the isolation of pure cPHA has become impossible. Unlike in bacteria, where the isolation of PHB is mostly based on the relative stability of the polymer to alkaline hypochlorite, the isolation of cPHB using the same protocol is highly unpredictable [19, 50]. Thus, as suggested by Reusch [19], it is generally advisable to quantify cPHB before attempting to separate or isolate it.

Reusch and Sadoff [47] first reported the isolation of cPHB from competent *E. coli*, where total PHB was extracted with cold CHCl_3 to yield $9.6 \mu\text{g/g}$ (wt/wt) wet mass of cPHB. Since then, only three pure cPHA have been successfully isolated. Seebach *et al.* [48] was able to extract cPHB from competent *E. coli* cells using an enhanced isolation procedure different from that used by Reusch and Sadoff [47]. Here, the pelleted cells were freeze-dried and the residue was extracted with dry CHCl_3 , saturated with cold (4°C) SDS. The remaining insoluble material was boiled for a long period in CHCl_3 . The extracts (both hot and cold) obtained afterward were purified by a Bio-Rad S-X3 bead treatment and further analyzed in a high-field NMR spectrometer. Both extracts showed signals of P(3-HB) with the hot extract containing significantly higher amounts. The hot extract was further purified by its continuous injection into a GPC system. Through this, chain lengths of cPHB were roughly

assigned [48]. Seebach *et al.* [48] researched further to isolate cPHB component from spinach, beef-heart, human aortae, and mitochondria to obtain H-NMR detectable amounts. Through this study, it was established that cPHB extracted from *E. coli* and spinach leaves is composed of (*R*)-3-hydroxybutanoic acid moieties [48].

Further isolation of eukaryotic cPHA in its pure state from sugar beet (*Beta vulgaris L.*) has been described [49]. In this study two different methods were used to yield 500 µg (80 g beet powder) and 600 µg (100 g beet powder) of pure cPHA respectively (see [Figure 4.1](#)).

The highest cPHA yield was observed using method 2. This was probably due to the addition of a partition procedure where the methanol precipitate was partitioned between chloroform and water (2:1) and this must have led to further reduction of impurities.

It is important to note that for the isolation of low concentrations of cPHB from these samples, large amounts of samples were required.

4.2.7 PHA Oligomers in Eukaryotes

It is well known that sPHB is a ubiquitous biopolymer confined to certain prokaryotic bacteria. Low molecular weight PHB found complexed to other macromolecules (cPHB) has also been shown to be widely distributed in representative organisms of nearly all biological cells and phyla [51]. While it's been reported that PHA oligomers are present in all living compartments of prokaryotic cells, it has, however, only been extensively examined in the Prokaryotic cell, *Escherichia coli*. Wild type *E. coli* is itself not capable of producing sPHA under normal growth conditions, but it has been found capable of producing small amounts of low molecular weight PHB complexed to cell membranes within its plasma membranes and not within its cytosols [47].

Reusch [5] was the first to isolate this complex form of PHB from the cell membrane of competent *E. coli* while examining transitions in lipid phase membranes of *Azotobacter vinelandii* and *Bacillus subtilis* cells during the development of genetic competence. The increase in transformability was followed by the occurrence of a sharp and irreversible

fluorescence peak corresponding to an increase in the concentration of PHB in the plasma membranes of *E. coli*. Naturally, PHB is not known to form intramolecular bonds and thus not capable of forming the quasi-crystalline structure on its own as indicated by the sharp fluorescence peak. As a result, the PHB was thought to be complexed and its complexant was assumed to be water-soluble due to a significant decrease in peak intensity when the cells were washed, especially with chelating buffers [5, 23]. PHB extracted from *E. coli* contained the anionic polymer PPi and Ca^{2+} which were surmised to be complexants of PHB since these hydrophilic moieties are greatly insoluble in chloroform [51]. Furthermore, it was discovered in Reusch's study [51], that this oligomer is located in the inner membrane of *E. coli*, where it occurs together with the calcium salt of inorganic polyP. The structure, composition, and distribution of the cPHB suggest that it may play a role in the regulation of intracellular calcium and in calcium signaling, as well as permitting calcium, DNA, or phosphate transport across its inner membrane [52]. Since then, *E. coli* has become the most preferred organism for cPHB studies. This is because it does not form inclusion bodies with sPHB that would otherwise have been difficult to separate from low molar mass PHB.

Huang and Reusch [20] further studied the presence of PHB in *E. coli* and discovered that the PHB in this cell is found all through the cells complexed to CaPolyP_i and primarily associated with proteins. Furthermore, it was detected in this study that the properties of the protein-associated PHB were different from those of cellular inclusion sPHB. More than 80% of the PHB detected in *E. coli* were found in the cytoplasm, with the greatest concentration being observed in the ribosomal fraction [20, 53].

In another study by Tsuge *et al.* [54], low molar mass PHA was synthesized by *R. eutropha* engineered with recombinant plasmid pBBREE32d13 carrying PHA synthase of *Aeromonas caviae*. Marine *Bacillus subtilis* and *Streptomyces lividans* were also detected to produce oligo-PHB in associations with calcium ions and polyP [55].

Since the regulation of calcium transport is being viewed as an important physiological function in bacteria, and PolyP_i synthesis is also known to be widely distributed in eukaryotic systems, it was therefore important to investigate if cPHB also exists in eukaryotic membranes. To this end, there have been quite a few studies [49, 50, 52] on detecting the presence of cPHB in eukaryotic cells to which a number of eukaryotic cells have been investigated to synthesize and accumulate cPHB. These cells include yeast (*Saccharomyces cerevisiae*), sugar beet (*Beta vulgaris L.*), peanut, cat muscles, sheep (intestine), and spinach, further showing that oligomeric PHB has excellent biocompatibility in animals [22, 55, 56]. In these studies, the PHBs form complexes with polyphosphate, various lipids, or proteins and play a role in membrane transport, thus making its solubility performance unpredictable [48, 52, 57].

A survey carried out on plant and animal tissues by Reusch [52] showed that PHB is indeed synthesized by a wide variety of eukaryotic cells, further illustrating that PHB is nearly ubiquitous in all cells. The long-chain-length PHB with high dispersity that forms inclusion bodies in some bacteria was not found in these studied eukaryotic cells. Meanwhile, it was short-chain-length PHB associated with CapolyP_i that was omnipresent in these cells. Just like in *E.coli* and other reported bacteria known to accumulate cPHB, cPHB was also found to be in membrane fractions of these eukaryotes, as indicated by the intracellular distribution of this complex in bovine liver [52], although the complex formed was primarily in the mitochondria and microsomes. The eukaryotic polyP_i chain length was a little greater (170–220) than that of bacterial PolyP_i (130–170), although the eukaryotic cPHB had the same broad range of chain lengths (120–200 subunits) as those in bacterial membranes. As suggested by the ubiquitous nature of the complex, the structure of PHB-Ca-polyP_i has an important physiological function. Furthermore, the intracellular distribution of the complex, its composition, and its structure also suggest its involvement in the storage and transport of

Ca^{2+} and PO_4^{2-} , giving it the function of regulating intracellular Ca^{2+} and transmitting Ca^{2+} signals [52].

Previous work by Reusch [19] also showed that cPHB is present in minute quantities in human blood plasma (predominantly associated with low-density lipoproteins (LDL), human aorta, bovine heart, liver mitochondria, and bovine serum albumin [19, 48, 58]. Seebach *et al.* [48] also reported their study on the isolation of cPHB from several eukaryotic organisms including yeast, spinach leaves, celery leaves, celery stalk, chicken liver, beef liver, lamb heart, beef heart, and beef brain.

To obtain more information on eukaryotic cPHA-biosynthesis mechanisms, Suzuki *et al.* [49] isolated a cPHA-component obtained from a complex with Ca-polyP from sugar beet (*Beta vulgaris L.*) and further determined its structure. This complex was discovered to be a homopolymer comprised of 3-hydroxybutyrate. Using MALDI MS, the number average molar mass and dispersity index of the isolated cPHA were determined to be $M_n = 9\,100$ Da and $\bar{D} = 1.01$ respectively, indicating that beet cPHA has a somewhat lower molar mass than the already known cPHA of *Escherichia coli*. Beet cPHB had a shorter chain PHB of $n = 106$ with a much lower 3HV content than cPHA from *E. coli*. The structural analysis of the obtained cPHA in this study also revealed that 100 mol-% of the carboxyl end is free, about 30 mol-% of the hydroxyl end is also free with about 70 mol-% of this end being masked, and the hydroxyl end group is also masked by at a minimum six identified short-chain alkanedioic and alkanolic acids [49]. These findings confirmed the organism-dependent structural diversity of cPHA (see also [Figure 4.2](#)).

Suzuki *et al.* [21] also reported his findings from the isolation of cPHA from commercial baker's yeast. In his study, 2.6 mg of pure cPHA (referred to as yeast cPHA-1) was isolated from 850 g dry weight of the yeast cells, making it the second cPHA being purified from eukaryotic organisms. The cPHA-1 obtained was further characterized using ^1H -NMR which

revealed four comonomers; 3-HV, 4-hydroxybutyrate (4-HB), 3-hydroxypropanoate (3-HP), and crotonate (CA). The presence of 3-HB, 3-HV, and very small units of CA was also confirmed by further GC-MS of cPHA-1 ethanolyzates. The amount of each comonomer in cPHA-1 was determined to be 1.03 mol-% of 3HV, 0.06 mol-% of 4-HB, 0.07 mol-% of 3-HP, and 0.02 mol-% of CA. Notably, as observed in this study, yeast cPHA-1 had an almost identical monomer content to beet cPHA (0.1 mol-% 4-HB, 0.01 mol-% CA) but had a ca. 10-fold greater 3HV content than beet cPHA [21]. The M_n of cPHA-1 was observed to be 57.4, which is much shorter than the chain length observed for beet cPHA. Significant signals indicating free-end 3-hydroxyl and carboxyl groups of cPHA-1 were also identified by the ^{31}P NMR spectrum [21]. The content of these groups corresponded to 17.5% of the molecules for the hydroxyl groups and 3.6% of the molecules for the carboxyl groups. These values also suggested the presence of cyclic molecules. It was also discovered in this study that changing the culture medium could directly influence the molecular weight and not the polydispersity of cPHA from baker's yeast.

4.2.8 PHA Oligomers for the Structural Studies of Their Precursors by Mass Spectrometry

The microstructure of high-molecular-weight bacterial PHA has been originally determined by ^{13}C NMR based on diad and triad analysis. However, the significant information on PHA copolymers microstructure was provided by mass spectrometry. For this purpose, the theoretical mass spectra of PHA copolymers containing various compositions of repeat units were generated, and compared with those experimentally acquired with the aid of such “soft” MS ionization techniques as matrix-assisted laser desorption-ionization (MALDI), fast atom bombardment (FAB), desorption-chemical ionization (DCI), and electrospray-ionization mass spectrometry (ESI-MS) [59]. Moreover, the electrospray ionization multistage mass spectrometry (ESI-MSⁿ) has been successfully applied for verification of PHA microstructure. By means of these methodologies, the random comonomer arrangements have been confirmed in poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), (PHBV), poly(3-

hydroxybutyrate-*co*-3-hydroxyhexanoate), (PHBH), copolymers, and recently for PHA with more than two different repeat units [60].

The ESI-MS/MS structural studies of bacterial PHA copolymers were based on the analysis of their oligomers obtained either by alkaline hydrolysis or controlled moderate-temperature degradation induced by carboxylate moieties. Using both of these approaches, oligomers containing carboxylic and olefinic end groups and the same composition and sequence distribution as the starting materials were obtained as revealed by ^1H -NMR, ^{13}C NMR, and ESI-MS/MS analysis [61].

4.2.9 Functionalization of PHA Oligomers Contained Unsaturated End Groups

PHA can be chemically modified to produce oligomers with the introduction of functional groups depending on the tailored functionality. So far, there have been several publications related to the various chemical modifications that can be employed for the production of synthetic oligomers [7, 26, 28].

4.2.10 Thermal Degradation

This is the most widely employed degradation process used for the production of synthetic PHA oligomers. It is the degradation of polymer chains due to overheating, where at high temperatures, this process results in volatile monomers, dimers, and trimers [7]. At moderately low temperatures (170–200°C), the degradation of PHA results in the production of a well-defined oligomer (usually 500–10 000g/mol macromolecule) with an unsaturated group (predominantly a *trans*-alkenyl end group) and a carboxylic group. In Yu's [62] patent, a controlled thermal degradation method was described to produce desired and well defined low molar mass PHA with functional end groups. In this study, a chemically stable substance (e.g., diethylene glycol, poly(ethylene glycol) or tetraethylene glycol) with a high boiling point of about 250°C was added to the reaction process to serve as a solvent or diluter during the thermal degradation process of high molar mass PHA. With this method, the window to

obtain PHA oligomers with controlled molar mass is large, so that the degradation process occurs in a better controlled manner, with improved heat transfer and more efficient stirring. Due to the large reaction period, a desirable molar mass can be achieved [62].

4.2.11 Controlled Moderate-Temperature Degradation

Moderate-temperature degradation of poly(3-hydroxyalkanoate)s (3-PHA) can be induced by carboxylate groups according to E1cB mechanism (see Figure 4.3). In the case of 3-PHB with end groups in the form of carboxylic acid salts with Na^+ , K^+ , and Bu_4N^+ counterions, the degradation induced by intermolecular α -deprotonation by carboxylate was suggested. It was assumed that this process is the main 3-PHA decomposition pathway at moderate temperatures. Oligomers containing carboxylic and unsaturated end groups were formed in this way [63].

Moreover, the degradation of 3-PHA with selected salts of organic and mineral acids was investigated. The significant decrease in 3-PHA thermal stability in the presence of salts of weak Bronsted-Lowry acids was observed due to an anionic degradation reaction proceeding via an E1cB mechanism. Furthermore, continuous poly(3-hydroxybutyrate) controlled degradation was developed by a moderate-temperature process using carbonic acid salts as “initiators” of moderate-temperature degradation. Foamed 3-PHA oligomeric macromonomers, were obtained by a reactive extrusion process [64]

Furthermore, bacterial (P(3HB4HB)) degradation under mild reaction conditions induced by carboxylate salts leads to the thermal degradation of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) and allows the obtaining of uniform linear oligomers. Moderate reaction temperature conditions and the presence of carboxylate salt allow for avoiding of the degradation process through a back-biting reaction which occurs even when a small amount of 4HB repeating units is present [65].

4.2.12 Thermooxidative Degradation of PHB

The thermal treatment of PHB in an oxygen/ozone mixture resulted in an increased rate of polymer backbone scission. The non-volatile degradation products contained macromolecules with several types of terminal groups, but also a part of the 3-hydroxybutyrate repeating units was transformed into 3-malic acid units. NMR and multistage MS characterization revealed the random distribution of 3-malic acid units in the oligomeric products as well as the content of the malic acid units being dependent on oxidation conditions [66].

4.2.13 Microwave-Assisted Degradation and UV Irradiation

Recently, microwave-assisted degradation of PHA has been employed as an inexpensive degradation route to PHA oligomers, since this method generates an efficient internal heating system by direct coupling of microwave energy with molecules [67, 68]. Here, sPHA samples are degraded in a microwave synthesis reactor operated at a desired power and temperature levels [9]. Ramier *et al.* [9] used this approach at 200°C–220°C to obtain high yields of oligomers ($M_n \leq 1\,000$ g/mol) characterized by a carboxyl group at one chain end and terminal crotonic ones on the other side. The obtained oligomeric PHA was also characterized by fluctuating molar masses in the shortest of times possible, about a 100 times faster rate than those obtained with conventional thermal degradation. This study further established that two microwave-assisted procedures could be successfully used: the first is irradiation with a constant temperature to obtain PHA oligomers with high yields and molar masses lower than 1 000 g/mol and the second is irradiation under constant power to afford oligoesters with higher molar masses [9]. Shangguan *et al.* [69] made use of UV irradiation to obtain oligomers with reactive radical groups after the quick controlled degradation of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) [69, 70]

4.2.14 Acid/Base Catalyzed Hydrolysis/Methanolysis of PHA

In this method, PHA are subjected to hydrolysis in either acid or base, leading to a slow and well-controlled depolymerization of the aliphatic polyester chains. Timbart *et al.* [16] have reported the synthesis of well-defined *mcl*-PHA oligomers (Poly(3-hydroxyoctanoate)

oligomers – PHO) from natural *mcl*-PHA using a basic hydrolysis, acid-catalyzed reaction with para-toluenesulfonic acid monohydrate (APTS) and methanolysis catalyzed by H₂SO₄. The ¹H NMR spectrum showed that the PHO oligomers produced by basic degradation at pH = 14 contained an unsaturated end group. However, more efficient production of PHO oligomers was observed with acid-catalyzed reaction and methanolysis. A MALDI-TOF spectra of PHO oligomers obtained with acid-catalyzed reaction showed the formation of linear oligomers having a hydroxyl group at one end and a carboxylic group at another end, with a very low proportion of cyclic structure. When Lukasiewicz and his colleagues [26] recently used an acid hydrolysis method, oligomeric PHA was obtained from natural *mcl*-PHA. The plasticizing effect of the obtained oligomeric PHA on natural PHB was also studied via characterization of mechanical and thermal properties of the blends during the course of aging at varying ambient conditions. The oligomeric PHA obtained in this study was further confirmed as a suitable biodegradable and biocompatible plasticizer for natural PHB, where plasticizing of PHB with oligomeric PHA resulted in a softer and more flexible PHB biomaterial [26].

4.2.15 Functionalization of PHA to Oligomers Containing Hydroxyl End Groups

Degradation of PHA via a reduction reaction with lithium borohydride leads to oligo(hydroxyalkanoate) diols (see [Figure 4.4](#)):

The structural characterization of the oligomers conducted using NMR and ESI-MSⁿ analyses confirmed that oligomers were terminated by two hydroxyl end groups. The reduction of the PHA occurred in a statistical way, regardless of the chemical structure of the comonomer units or of the microstructure of the polyester chain [71, 72]. This method can be used to synthesize various PHA oligodiols that were useful in the further synthesis of tailor-made biodegradable materials [73, 74].

4.2.17-6 Bioactive Oligomers of PHA

In a transesterification reaction, an alcohol molecule and an ester molecule react in the presence of acid; basically, one ester is transformed into another ester. This process leads to the synthesis of oligomers with either carboxyl or hydroxyl functionalities. Using this transesterification reaction, Kwiecień *et al.* [75] were able to develop two methods for the preparation of pesticide-oligomer conjugates. In the first method (one-pot), an MCPA-oligo (3HB-*co*-4HB) conjugate was synthesized using a transesterification reaction of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) and P(3HB-*co*-4HB) biopolyester with (4-chloro-2-methylphenoxy) acetic acid (MCPA) in the presence of 4-toluenesulfonic acid monohydrate (see Figure 4.5).

Oligo (3HB)-tyrosol conjugate was synthesized using a two-step approach. In the first approach, cyclic oligomers were obtained via a ring-closing depolymerization reaction and the cyclic oligomers were reacted with 4-(2-hydroxyethyl) phenol (tyrosol) in the presence of lipase from *Candida antarctica* (see Figure 4.6).

The synthetic route to prepare a PHB-amine conjugate containing a hydrolyzable imine bond was also reported. A short-chain PHB crotonate obtained by moderate-temperature degradation of natural polyester was converted into PHB glyoxylate via ozonolysis followed by reductive decomposition of peroxidic products with dimethylsulfide. Aldehyde-functionalized PHB was obtained quantitatively without polymer backbone degradation. The aldehyde-functionalized PHB can be a valuable biocompatible carrier for novel drug delivery systems [76].

4.2.187 Copolymers Containing PHA Building Blocks

Low molar mass macroinitiators derived from natural PHA, containing unsaturated and activated by 18-crown-6 ether carboxylic end groups, were used in anionic ring-opening polymerization (ROP) of racemic β -butyrolactone, and new diblock copolymers of selected PHA (PHB, PHBV, PHO) with atactic poly[(R,S)-3-hydroxybutyrate] (aPHB) were obtained.

The suitability of these polymeric materials for cardiovascular engineering and as blend compatibilizers was demonstrated [77].

Block copolyesters containing PHA and poly(D,L-lactide) structural units were obtained with a microwave-assisted process [9]. The PHA oligomers used for this purpose were obtained by acid-catalyzed methanolysis of corresponding native PHA.

The amphiphilic hyaluronan (HA) grafted with poly(3-hydroxyalkanoates) (PHA) oligomers, were obtained by a “grafting to” strategy [28]. PHA oligomers containing carboxylic terminal moieties were prepared by partial hydrolysis of PHA in acetic acid/water system. Such graft copolymers can be physically loaded with hydrophobic drugs and may serve as drug delivery systems.

Nguyen [78] had earlier suggested that PHB oligomers with carboxylic acid terminal group could be further subjected to modifications and graft polymerization through a readily available radical mechanism in acrylic backbone and PHB side chain copolymers.

4.3 Oligomers of Synthetic Analogs of Natural PHA

4.3.1 Monodisperse Synthetic Analogs of PHA

Monodisperse oligomers of PHA, up to the 128 repeat units (molecular mass ca. 11 000 Da, as revealed by MALDI MS) were synthesized by fragment coupling (see [Figure 4.7](#)).

With these oligomers it was possible to calibrate the standards used for molar mass determinations of PHB by gel-permeation chromatography (GPC).

Using the same methodology as for the *isotactic* monodisperse PHB the *syndiotactic*, *atactic*, and *block* oligomers of PHB, with a given sequence of (*R*) and (*S*)-configuration of the 3HB units along the chain were prepared. Such non-natural PHA oligomers are important for studying the stereoselectivity of enzymatic PHB depolymerization [22].

4.3.2 Functional Oligomers of PHA Analogs

The facile synthesis of biomimetic predominantly isotactic oligo-(*R*)-3-hydroxybutyrate by region-selective anionic polymerization of (*S*)- β -butyrolactone initiated with 3-hydroxyacid sodium salt/crown ether complexes was described [79].

Such a synthetic analog of natural PHB was used for the preparation of the models of artificial channels in cell membranes. Das *et al.* [80] found that complexes of biomimetic OHBs with M_n 1670 and \bar{D} 1.2 and with inorganic polyPs can be used to create serviceable artificial cation channels of limited divalent cation selectivity in planar bilayers.

Piddubniak *et al.* [81] described the chemical synthesis and toxicity studies of well-defined tailor-made oligo-[*R,S*]-3-hydroxybutyrates (OHBs). The results indicate potential applicability of these oligomers as drug delivery carriers. Several OHBs with a number average molar mass (M_n) ranging from 800 to 2 400 have been synthesized and tested on transformed hamster V79 fibroblasts and murine melanoma B16(F10) cells using the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT)-based drug resistance and clonogenic survival assays. It was shown that 96 h incubation of cells with 1–9 mg/ml of OHBs did not affect cell viability. Moreover, incubation of OHBs with rat hepatoma FTO-2B cells stably transfected with a chloramphenicol acetyltransferase (CAT) gene ligated to a heat-inducible hsp70i gene promoter demonstrated that OHBs did not induce a cellular stress response. Furthermore, they demonstrate that doxorubicin conjugated with OHB is effectively taken up by murine melanoma B16(F10) cells *in vitro* and localizes in the cytoplasm. These data show for the first time that tailor-made biodegradable and biocompatible oligomers of 3-hydroxybutyric acid can be taken into consideration as effective, non-toxic vectors for the delivery of drugs in a conjugated form [81].

Elustondo *et al.* [82] investigated the ability of aPHB to interact with living cells and isolated mitochondria and the effects of these interactions on membrane ion transport using a fluorescein derivative of aPHB. The obtained results indicated that PHB demonstrates

ionophoretic properties in biological membranes, and this effect is most profound in mitochondria due to the selective accumulation of the polymer in this organelle [82].

The transformation method of low molar mass crotonate-terminated poly(3-hydroxybutyrate)s (PHB), obtained by anionic ROP of β -butyrolactone [83] and controlled degradation of high-molar mass bacterial PHB, into mono- and di-epoxy-functionalized reactive poly(3-hydroxybutyrate)s is via oxidation of crotonate end groups to α -3-methyloxirane-2-carboxylates, while carboxylate terminal groups were functionalized by simple alkylation with epibromohydrin was described. Combining these two methods resulted in telechelic structures: α -3-methyloxirane-2-carboxylate- ω -glycidyl PHB. Moreover, the reactivity of 3-methyloxirane groups of a functional aPHB was confirmed in experiments with primary amine and primary alcohol, revealing very high yield in reaction with alcohol, while degradation of polymer backbone was noticed during reaction with amine [84].

4.3.3 Bioactive Oligomers of PHA Analogs

Synthetically prepared PHA oligomers were found to be non-toxic and they may be used as carriers covalently bounded to suitable bioactive compounds suitable for medical, cosmetic, agrichemical, and functional packaging applications.

The drug delivery systems were focused on penicillin G, acetylsalicylic acid, and ibuprofen [85, 86, 87]. Novel conjugates of the non-steroidal anti-inflammatory drug ibuprofen were synthesized by anionic ring-opening polymerization of (R,S)- β -butyrolactone initiated with an alkali metal salt of (S)-(*t*)-2-(4-isobutylphenyl) propionic acid (ibuprofen). Using the MTT cell proliferation assay, it was demonstrated that such conjugates exhibited significantly increased potential to inhibit proliferation of HT-29 and HCT 116 colon cancer cells when compared to free ibuprofen. Moreover, the conjugates of ibuprofen and OHB are less toxic as was shown in oral acute toxicity test in rats [87].

Incorporation of bioactive compounds into the β -lactones structure may lead to homo- and co-oligoesters with a bioactive moiety covalently linked as pendant groups along an oligomer backbone. This synthetic strategy was applied for preparation of the PHA synthetic analogs with ibuprofen pendant groups [88], pesticide moieties [32], and antioxidants used in cosmetics [89, 90, 91].

4.3.4 Copolymers Containing PHA Analogs

A synthetic aPHB oligomer possessing hydroxyl end groups, obtained in anionic polymerization of (*R,S*)- β -butyrolactone, was applied as a precursor for preparation of a coordination macroinitiator to be used for ϵ -caprolactone polymerization. The respective block copolymers were prepared in this way [92].

aPHB with a bishydroxy chain terminus was obtained through ring-opening polymerization of (*R, S*)- β -butyrolactone mediated by the activated anionic initiator 2,2-bis(hydroxymethyl)butyric acid (BHBA) tetrabutylammonium salt. Then, the polyester was modified to obtain a macroinitiator bifunctional species applicable in an ATRP process. Next, in an ATRP of methacrylic PEG macromonomer a water-soluble brush copolymer was synthesized [93].

Block copolymers of (*R, S*)- β -butyrolactone with pivalolactone (PVL) are prepared in order to define the effect of crystalline domains provided by poly(pivalolactone) on the biodegradability of atactic poly(β -butyrolactone), aPHB. The aPHB was synthesized from racemic β -butyrolactone, in the presence of a potassium alkoxide/18-crown-6 complex, and such a living polymer was applied for polymerization of PVL, yielding block copolymers, aPHB-*b*-PPVL, of tailored molecular weight and composition. While plain aPHB does not biodegrade, the biodegradation rate of aPHB-*b*-PPVL copolymers increases along with the increase of crystalline PPVL domains [94].

Graft copolymers were synthesized via an anionic grafting reaction of β -butyrolactone on poly(methyl methacrylate) (PMMA). Partially saponified PMMA bearing carboxylate anions complexed by 18-crown-6 potassium counterion acts as a macroinitiator of β -butyrolactone polymerization. As a result, graft polymers of PMMA with grafted poly(butyrolactone) side chains are produced in high yield over a wide graft composition range [95]. aPHB graft copolymers were also prepared via a macromonomer method, i.e., grafting through copolymerization [96]. Poly[(R,S)-3-hydroxybutyrate] telechelics were used for the synthesis of poly(methyl methacrylate)-*b*-poly(3-hydroxybutyrate) block copolymers [97]. Branched, aliphatic polyurethanes (PURs) were synthesized and compared to linear analogs. The influence of polycaprolactonetriol and synthetic aPHB oligomers in soft segments on structure, thermal, and sorptive properties of PURs was determined. It was found that increasing the aPHB amount in the structure of branched PURs reduced a tendency of urethane groups to hydrogen bonding. Thus, by controlling the number of branches and the amount of a-PHB in soft segments, thermal and absorptive properties of aliphatic PURs could be controlled [98].

4.4 Conclusions and Outlook

Polyhydroxyalkanoates (PHA) are a group of polyesters with excellent biodegradable and biocompatible properties. Additionally, their degradation products after hydrolysis are non-toxic, therefore making this biopolymer an attractive biomaterial for various end use applications. However, due to several undesirable physical properties of storage PHA (e.g., poor mechanical properties, poor solubility) and limited functionalities, the application of PHA in the other areas of importance is still limited. In recent years, significant efforts have been made to investigate a relationship between structure, properties, and function of advanced biodegradable polymeric materials in order to fulfill requirements for their specific novel applications in the future.

One way to increase the mechanical properties of PHA and expand their potential applications is to use PHA oligomers containing reactive functional end groups which could be used as building blocks for the preparation of new biopolymers with enhanced properties and high-value applications. PHA oligomers are low molecular weight PHA with a limited number of 3-hydroxyacids repeated units (max. 200 residual units). They are present in numerous intracellular fluids of all types of cells and in some cases, complexed to other cellular macromolecules including inorganic polyPs. They can also be synthesized either naturally in prokaryotic and eukaryotic cells or via several chemical modifications such as basic hydrolysis or transesterification. The chemical modification of storage PHA yields synthetic oligomers with the additional functional end groups, depending on the tailored functionality. These chemical modifications, such as controlled moderate-temperature degradation, thermal degradation, and thermo-oxidative degradation of PHB often leads to the synthesis of oligomers with unsaturated, carboxylic, or hydroxyl end groups. The PHA oligomers derived either from natural aliphatic polyesters or their synthetic analogs could be used as carriers covalently bound to bioactive compounds. Polymeric controlled-release delivery systems of bioactive compounds can provide novel opportunities suitable for various applications in medicine, cosmetic industry, food packaging, or agri-chemistry.

References

1. Lemoigne M. Production d'acide β -oxybutyrique par certaines bactéries du groupe du *Bacillus subtilis*. *Comptes Rendus de l'Académie des Sciences*. 1973; 176(1923): 1760–1761.
2. Koller M. Biodegradable and biocompatible polyhydroxy-alkanoates (PHA): Auspicious microbial macromolecules for pharmaceutical and therapeutic applications. *Molecules* 2018; 23(2): 362.
3. Jendrossek D, Pfeiffer D. New insights in the formation of polyhydroxyalkanoate granules (carbonosomes) and novel functions of poly (3-hydroxybutyrate). *Environ Microbiol* 2014; 16(8): 2357–2373.
4. Elustondo P, Zakharian E, Pavlov E. Identification of the polyhydroxybutyrate granules in mammalian cultured cells. *Chem Biodiv* 2012; 9(11): 2597–2604.

5. Reusch RN, Sadoff HL. D-(-)-poly-3-hydroxybutyrate in membranes of genetically competent bacteria. *J Bacteriol* 1983; 156(2): 778–788.
6. Martin DP, Peoples OP, Williams SF, *et al.* Nutritional and therapeutic uses of 3-hydroxyalkanoate oligomers. U.S. Patent 6,380,244. 2002.
7. Raza ZA, Riaz S, Banat IM. Polyhydroxyalkanoates: Properties and chemical modification approaches for their functionalization. *Biotechnol Prog* 2018; 34(1): 29–41.
8. Li Z, Loh XJ. Water soluble polyhydroxyalkanoates: Future materials for therapeutic applications. *Chem Soc Rev* 2015; 44(10): 2865–2879.
9. Ramier J, Grande D, Langlois V, *et al.* Toward the controlled production of oligoesters by microwave-assisted degradation of poly (3-hydroxyalkanoate)s. *Polym Degr Stab* 2012; 97(3): 322–328.
10. Adamus G, Kurcok P, Radecka I, *et al.* Bioactive oligomers from natural polyhydroxyalkanoates and their synthetic analogues. *Polimery* 2017; 62(4): 317–322.
11. Maksymiak M, Debowska R, Bazela K, *et al.* Designing of biodegradable and biocompatible release and delivery systems of selected antioxidants used in cosmetology. *Biomacromolecules* 2015; 16(11): 3603–3612.
12. Babinot J, Renard E, Langlois V. Controlled synthesis of well-defined poly (3-hydroxyalkanoate) s-based amphiphilic diblock copolymers using click chemistry. *Macromol Chem Phys* 2011; 212(3): 278–285.
13. Ajioka M, Suizu H, Higuchi C, *et al.* Aliphatic polyesters and their copolymers synthesized through direct condensation polymerization. *Polym Degr Stab* 1998; 59(1–3): 137–143.
14. Hiki S, Miyamoto M, Kimura Y. Synthesis and characterization of hydroxy-terminated [RS]-poly (3-hydroxybutyrate) and its utilization to block copolymerization with L-lactide to obtain a biodegradable thermoplastic elastomer. *Polymer* 2000; 41(20): 7369–7379.
15. Andrade AP, Neuenschwander P, Hany R, *et al.* Synthesis and characterization of novel copoly (ester-urethane) containing blocks of poly-[(R)-3-hydroxyoctanoate] and poly-[(R)-3-hydroxybutyrate]. *Macromolecules* 2002; 35(13): 4946–4950.
16. Timbart L, Renard E, Tessier M, *et al.* Monohydroxylated poly (3-hydroxyoctanoate) oligomers and its functionalized derivatives used as macroinitiators in the synthesis of degradable diblock copolyesters. *Biomacromolecules* 2007; 8(4): 1255–1265.
17. Klaerner G, Padmanabhan R. Multi-step/step-wise polymerization of well-defined oligomers. In: *Reference Module in Materials Science and Materials Engineering*. Hashmi S., Smithers G. Eds. Elsevier 2018.

18. Freier T, Kunze C, Nischan C, *et al.* *In vitro* and *in vivo* degradation studies for development of a biodegradable patch based on poly (3-hydroxybutyrate). *Biomaterials* 2002; 23(13): 2649–2657.
19. Reusch RN. Biological complexes of poly- β -hydroxybutyrate. *FEMS Microbiol Rev* 1992; 9(2–4): 119–129.
20. Huang R, Reusch RN. Poly (3-hydroxybutyrate) is associated with specific proteins in the cytoplasm and membranes of *Escherichia coli*. *J Biol Chem* 1996; 271(36): 22196–22202.
21. Suzuki Y, Esumi Y, Koshino H, *et al.* Characterization of short-chain poly-3-hydroxybutyrate in baker's yeast. *Phytochemistry* 2008; 69(2): 491–497.
22. Seebach D, Fritz MG. Detection, synthesis, structure, and function of oligo (3-hydroxyalkanoates): Contributions by synthetic organic chemists. *Int J Biol Macromol* 1999; 25(1–3): 217–236.
23. Reusch RN, Hiske TW, Sadoff HL. Poly- β -hydroxybutyrate membrane structure and its relationship to genetic transformability in *Escherichia coli*. *J Bacteriol* 1986; 168(2): 553–562.
24. Seebach D, Brunner A, Büger HM, *et al.* Channel-forming activity of 3-hydroxybutanoic-acid oligomers in planar lipid bilayers. *Helv Chim Acta* 1996; 79(2): 507–517.
25. Dai D, Reusch RN. Poly-3-hydroxybutyrate synthase from the periplasm of *Escherichia coli*. *Biochem Biophys Res Commun* 2008; 374(3): 485–489.
26. Lukasiewicz B, Basnett P, Nigmatullin R, *et al.* Binary polyhydroxyalkanoate systems for soft tissue engineering. *Acta Biomater* 2018; 71: 225–234.
27. Adaya L, Millán M, Peña C, *et al.* Inactivation of an intracellular poly-3-hydroxybutyrate depolymerase of *Azotobacter vinelandii* allows to obtain a polymer of uniform high molecular mass. *Appl Microbiol Biotechnol* 2018; 102(6): 2693–2707.
28. Huerta-Angeles G, Brandejsová M, Nigmatullin R, *et al.* Synthesis of graft copolymers based on hyaluronan and poly (3-hydroxyalkanoates). *Carbohydr Polym* 2017; 171: 220–228.
29. Shrivastav A, Kim HY, Kim YR. Advances in the applications of polyhydroxyalkanoate nanoparticles for novel drug delivery system. *BioMed Research International* 2013; 2013: 581684.
30. Utsunomia C, Saito T, Matsumoto KI, *et al.* Synthesis of lactate (LA)-based poly (ester-urethane) using hydroxyl-terminated LA-based oligomers from a microbial secretion system. *J Polym Res* 2017; 24(10): 167.
31. Sugiyama A, Kobayashi T, Shiraki M, *et al.* Roles of poly (3-hydroxybutyrate) depolymerase and 3HB-oligomer hydrolase in bacterial PHB metabolism. *Curr Microbiol* 2004; 48(6): 424–427.
32. Kwiecień I, Bałakier T, Jurczak J, *et al.* Molecular architecture of novel potentially bioactive (co) oligoesters containing pesticide moieties established by electrospray ionization multistage mass spectrometry. *Rapid Commun Mass Spectrom* 2015; 29(6): 533–544.

33. Guérin P, Renard E, Langlois V. Degradation of natural and artificial poly [(R)-3-hydroxyalkanoate]s: From biodegradation to hydrolysis. In: *Plastics from Bacteria*. Berlin, Heidelberg: Chen G. Ed., Springer 2010; pp. 283–321.
34. Kobayashi T, Uchino K, Abe T, *et al.* Novel intracellular 3-hydroxybutyrate-oligomer hydrolase in *Wautersia eutropha* H16. *J Bacteriol* 2005; 187(15): 5129–5135.
35. Tseng CL, Chen HJ, Shaw GC. Identification and characterization of the *Bacillus thuringiensis* phaZ gene, encoding new intracellular poly-3-hydroxybutyrate depolymerase. *J Bacteriol* 2006; 188(21): 7592–7599.
36. Gebauer B, Jendrosseck D. Assay of poly (3-hydroxybutyrate) depolymerase activity and product determination. *Appl Environ Microbiol* 2006; 72(9): 6094–6100.
37. Kobayashi T, Shiraki M, Abe T, *et al.* Purification and properties of an intracellular 3-hydroxybutyrate-oligomer hydrolase (PhaZ2) in *Ralstonia eutropha* H16 and its identification as a novel intracellular poly(3-hydroxybutyrate) depolymerase. *J Bacteriol* 2003; 185(12): 3485–3490.
38. Arikawa H, Sato S, Fujiki T, *et al.* A study on the relation between poly (3-hydroxybutyrate) depolymerases or oligomer hydrolases and molecular weight of polyhydroxyalkanoates accumulating in *Cupriavidus necator* H16. *J Biotechnol* 2016; 227: 94–102.
39. Handrick R, Reinhardt S, Kimmig P, *et al.* The “intracellular” poly (3-hydroxybutyrate)(PHB) depolymerase of *Rhodospirillum rubrum* is a periplasm-located protein with specificity for native PHB and with structural similarity to extracellular PHB depolymerases. *J Bacteriol* 2004; 186(21): 7243–7253.
40. Saegusa H, Shiraki M, Kanai C, *et al.* Cloning of an intracellular poly [d (-)-3-hydroxybutyrate] depolymerase gene from *Ralstonia eutropha* H16 and characterization of the gene product. *J Bacteriol* 2001; 183(1): 94–100.
41. Abe T, Kobayashi T, Saito T. Properties of a novel intracellular poly (3-hydroxybutyrate) depolymerase with high specific activity (PhaZd) in *Wautersia eutropha* H16. *J Bacteriol* 2005; 187(20): 6982–6990.
42. Agus J, Kahar P, Hyakutake M, *et al.* Unusual change in molecular weight of polyhydroxyalkanoate (PHA) during cultivation of PHA-accumulating *Escherichia coli*. *Polym Degrad Stab* 2010; 95(12): 2250–2254.
43. Zaheer MR, Kuddus M. PHB (poly-β-hydroxybutyrate) and its enzymatic degradation. *Polym Adv Technol* 2018; 29(1): 30–40.
44. Volova TG, Prudnikova SV, Vinogradova ON, *et al.* Microbial degradation of polyhydroxyalkanoates with different chemical compositions and their biodegradability. *Microb Ecol* 2017; 73(2): 353–367.

45. Jendrossek D. Microbial degradation of polyesters. *Adv. Biochem. Eng. Biotechnol.* 2001; 71: 293–325.
46. Handrick R, Reinhardt S, Jendrossek D. Mobilization of poly (3-hydroxybutyrate) in *Ralstonia eutropha*. *J Bacteriol* 2000; 182(20): 5916–5918.
47. Reusch RN, Sadoff HL. Putative structure and functions of a poly- β -hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. *PNAS* 1988; 85(12): 4176–4180.
48. Seebach D, Brunner A, Bürger HM, *et al.* Isolation and ¹H-NMR Spectroscopic identification of poly(3-hydroxybutanoate) from prokaryotic and eukaryotic organisms: Determination of the absolute configuration (R) of the monomeric unit 3-hydroxybutanoic acid from *Escherichia coli* and Spinach. *Eur J Biochem* 1994; 224(2): 317–328.
49. Suzuki Y, Esumi Y, Koshino H, *et al.* Isolation and structure determination of complexed poly (3-hydroxyalkanoate) from beet (*Beta vulgaris* L.). *Macromol Biosci* 2005; 5(9): 853–862.
50. Law JH, Slepecky RA. Assay of poly- β -hydroxybutyric acid. *J Bacteriol* 1961; 82(1): 33–36.
51. Reusch RN. Low molecular weight complexed poly (3-hydroxybutyrate): A dynamic and versatile molecule *in vivo*. *Can J Microbiol* 1995; 41(13) Supplement 1: 50–54.
52. Reusch RN. Poly- β -hydroxybutyrate/calcium polyphosphate complexes in eukaryotic membranes. *Proc Soc Exp Biol Med* 1989; 191(4): 377–381.
53. Nobes GA, Maysinger D, Marchessault RH. Polyhydroxyalkanoates: Materials for delivery systems. *Drug Deliv* 1998; 5(3): 167–177.
54. Tsume T, Watanabe S, Sato S, *et al.* Variation in copolymer composition and molecular weight of polyhydroxyalkanoate generated by saturation mutagenesis of *Aeromonas caviae* PHA synthase. *Macromol Biosci* 2007; 7(6): 846–854.
55. Reusch RN. Streptomyces lividans potassium channel contains poly-(R)-3-hydroxybutyrate and inorganic polyphosphate. *Biochemistry* 1999; 38(47): 15666–15672.
56. Kavitha G, Rengasamy R, Inbakandan D. Polyhydroxybutyrate production from marine source and its application. *Int J Biol Macromol* 2018; 111: 102–108.
57. Abd-El-haleem D, Amara A, Zaki S, *et al.* Biosynthesis of biodegradable polyhydroxyalkanotes biopolymers in genetically modified yeasts. *Int J Environ Sci Technol* 2007; 4(4): 513–520.
58. Müller HM, Seebach D. Poly (hydroxyalkanoates): A fifth class of physiologically important organic biopolymers? *Angew Chem Int Ed* 1993; 32(4): 477–502.
59. Adamus G, Sikorska W, Kowalczyk M, *et al.* Sequence distribution and fragmentation studies of bacterial copolyester macromolecules: Characterization of PHBV macroinitiator by electrospray ion-trap multistage mass spectrometry. *Macromolecules* 2000; 33(16): 5797–5802.

60. Johnston B, Radecka I, Chiellini E, *et al.* Mass spectrometry reveals molecular structure of polyhydroxyalkanoates attained by bioconversion of oxidized polypropylene waste fragments. *Polymers* 2019; 11(10): 1580.
61. Kowalczyk M, Adamus G. Mass spectrometry for the elucidation of the subtle molecular structure of biodegradable polymers and their degradation products. *Mass Spectrom Rev* 2016; 35(1): 188–198.
62. Yu GE. Process of producing low molecular weight poly (hydroxyalkanoate)s from high molecular weight poly (hydroxyalkanoate)s. U.S. Patent 7,361,725. 2008.
63. Kawalec M, Adamus G, Kurcok P, *et al.* Carboxylate-induced degradation of poly (3-hydroxybutyrate)s. *Biomacromolecules* 2007; 8(4): 1053–1058.
64. Kawalec M, Sobota M, Scandola M, *et al.* A convenient route to PHB macromonomers via anionically controlled moderate-temperature degradation of PHB. *J Polym Sci Part A: Polym Chem* 2010; 48(23): 5490–5497.
65. Kwiecień M, Kawalec M, Kurcok P, *et al.* Selective carboxylate induced thermal degradation of bacterial poly (3-hydroxybutyrate-co-4-hydroxybutyrate)—Source of linear uniform 3HB4HB oligomers. *Polym Degrad Stab* 2014; 110: 71–79.
66. Michalak M, Kwiecień M, Kawalec M, *et al.* Oxidative degradation of poly (3-hydroxybutyrate). A new method of synthesis for the malic acid copolymers. *RSC Adv* 2016; 6(16): 12809–12818.
67. Kappe CO. Controlled microwave heating in modern organic synthesis. *Angew Chem Int Ed* 2004; 43(46): 6250–6284.
68. Galema SA. Microwave chemistry. *Chem Soc Rev* 1997; 26(3): 233–238.
69. Shangguan YY, Wang YW, Wu Q, *et al.* The mechanical properties and *in vitro* biodegradation and biocompatibility of UV-treated poly (3-hydroxybutyrate-co-3-hydroxyhexanoate). *Biomaterials* 2006; 27(11): 2349–2357.
70. Gumel, AM, Aris, MH, Annuar, MSM. Modification of polyhydroxyalkanoates (PHAs). In: *Polyhydroxyalkanoate (PHA) Based Blends, Composites and Nanocomposites*. Roy i., Visakh P M, Eds., RSC, 2014. pp. 141–182.
71. Kwiecień M, Adamus G, Kowalczyk M. Selective reduction of PHA biopolyesters and their synthetic analogues to corresponding PHA oligodiols proved by structural studies. *Biomacromolecules* 2013; 14(4): 1181–1188.
72. Chaber P, Kwiecień M, Zięba M, *et al.* The heterogeneous selective reduction of PHB as a useful method for preparation of oligodiols and surface modification. *RSC Adv* 2017; 7(56): 35096–35104.
73. Michalak M, Kwiecień I, Kwiecień M, *et al.* Diversifying polyhydroxyalkanoates—end-group and side-chain functionality. *Curr Org Synth* 2017; 14(6): 757–767.

74. Kwiecień M, Kwiecień I, Radecka I, *et al.* Biocompatible terpolyesters containing polyhydroxyalkanoate and sebacic acid structural segments- synthesis and characterization. *RSC Adv* 2017; 7: 20469–20479.
75. Kwiecień I, Radecka I, Kowalczyk M, *et al.* Transesterification of PHA to oligomers covalently bonded with (bio)active compounds containing either carboxyl or hydroxyl functionalities. *PLoS One* 2015; 10(3): e0120149.
76. Michalak M, Marek AA, Zawadiak J, *et al.* Synthesis of PHB-based carrier for drug delivery systems with pH-controlled release. *Eur Polym J* 2013; 49(12): 4149–4156.
77. Adamus G, Sikorska W, Janeczko H, *et al.* Novel block copolymers of atactic PHB with natural PHA for cardiovascular engineering: Synthesis and characterization. *Eur Polym J* 2012; 48(3): 621–631.
78. Nguyen S. Graft copolymers containing poly (3-hydroxyalkanoates)—A review on their synthesis, properties, and applications. *Can J Chem* 2008; 86(6): 570–578.
79. Jedliński Z, Kurcok P, Lenz RW. First facile synthesis of biomimetic poly-(R)-3-hydroxybutyrate via regioselective anionic polymerization of (S)- β -butyrolactone. *Macromolecules* 1998; 31(19): 6718–6720.
80. Das S, Kurcok P, Jedlinski Z, *et al.* Ion channels formed by biomimetic oligo-(R)-3-hydroxybutyrates and inorganic polyphosphates in planar lipid bilayers. *Macromolecules* 1999; 32(26): 8781–8785.
81. Piddubnyak V, Kurcok P, Matuszowicz A, *et al.* Oligo-3-hydroxybutyrates as potential carriers for drug delivery. *Biomaterials* 2004; 25(22): 5271–5279.
82. Elustondo PA, Angelova PR, Kawalec M, *et al.* Polyhydroxybutyrate targets mammalian mitochondria and increases permeability of plasmalemmal and mitochondrial membranes. *PLoS One* 2013; 8(9): e75812.
83. Kurcok P, Matuszowicz A, Jedliński Z. Anionic polymerization of β -lactones initiated with potassium hydride. A convenient route to polyester macromonomers. *Macromol Rapid Commun* 1995; 16(3): 201–206.
84. Michalak M, Kawalec M, Kurcok P. Reactive mono-and di-epoxy-functionalized poly (3-hydroxybutyrate)s. Synthesis and characterization. *Polym Degrad Stab* 2012; 97(10): 1861–1870.
85. Adamus G, Kowalczyk M. Electrospray multistep ion trap mass spectrometry for the structural characterisation of poly [(R, S)-3-hydroxybutanoic acid] containing a β -lactam end group. *Rapid Commun Mass Spectrom* 2000; 14(4): 195–202.
86. Juzwa M, Rusin A, Zawidlak-Węgrzyńska B, *et al.* Oligo(3-hydroxybutanoate) conjugates with acetylsalicylic acid and their antitumour activity. *Eur J Med Chem* 2008; 43: 1785–1790.

87. Zawidlak-Węgrzyńska B, Kawalec M, Bosek I, *et al.* Synthesis and antiproliferative properties of ibuprofen–oligo (3-hydroxybutyrate) conjugates. *Eur J Med Chem* 2010; 45(5): 1833–1842.
88. Kowalczyk M, Adamus G, Kwiecień I, *et al.* New biodegradable (co)polyesters from β -lactone monomers containing biologically active substances. Patent No. PL-225906. 2016.
89. Maksymiak M, Bałakier T, Jurczak J, *et al.* Bioactive (co) oligoesters with antioxidant properties—synthesis and structural characterization at the molecular level. *RSC Adv* 2016; 6(62): 57751–57761.
90. Maksymiak M, Dębowska R, Jelonek K, *et al.* Structural characterization of biocompatible lipoic acid – oligo(3-hydroxybutyrate) conjugates by ESI-mass spectrometry. *Rapid Commun. Mass Spectrom.* 2013; 27(7): 773–783.
91. Maksymiak M, Kowalczyk M, Adamus G. Electrospray tandem mass spectrometry for the structural characterization of p-coumaric acid - oligo(3-hydroxybutyrate) conjugates. *Int J Mass Spectrom* 2014; 359: 6–11.
92. Kurcok P, Dubois P, Sikorska W, *et al.* Macromolecular engineering of lactones and lactides. 24. Controlled synthesis of (R, S)- β -butyrolactone-*b*- ϵ -caprolactone block copolymers by anionic and coordination polymerization. *Macromolecules* 1997; 30(19): 5591–5595.
93. Koseva NS, Novakov CP, Rydz J, *et al.* Synthesis of aPHB-PEG brush co-polymers through ATRP in a macroinitiator–macromonomer feed system and their characterization. *Des Monomers Polym* 2010; 13(6): 579–595.
94. Scandola M, Focarete ML, Gazzano M, *et al.* Crystallinity-induced biodegradation of novel [(R, S)- β -butyrolactone]-*b*-pivalolactone copolymers. *Macromolecules* 1997; 30(25): 7743–7748.
95. Kowalczyk M, Adamus G, Jedlinski Z. Synthesis of new graft polymers via anionic grafting of β -butyrolactone on poly(methyl methacrylate). *Macromolecules* 1994; 27(2): 5720–5725.
96. Neugebauer D, Rydz J, Goebel I, *et al.* Synthesis of graft copolymers containing biodegradable poly(3-hydroxybutyrate) chains. *Macromolecules* 2007; 40(5): 1767–1773.
97. Alli A, Hazer B, Adamus G, *et al.* Polyhydroxyalkanoates/polyhydroxybutyrates (PHAs/PHBs). In: Guillaume SM, Ed., *Handbook of Telechelic Polyesters, Polycarbonates, and Polyethers*. Singapore: Pan Stanford Publishing 2017; pp. 65–113.
98. Brzeska J, Elert A, Morawska M, *et al.* Branched polyurethanes based on synthetic polyhydroxybutyrate with tunable structure and properties. *Polymers* 2018; 10(8): 826.

Figure 4.1 Isolation and purification outline of cPHA from sugar beet [49].

Figure 4.2 Chemical structures of beet cPHA and *E. coli* cPHA [49]

Figure 4.3 E1cB PHB degradation mechanism [63].

Figure 4.4 Reductive degradation of PHA [71].

Figure 4.5 Transesterification reaction of P(3HB-*co*-4HB) by MCPA [75].

Figure 4.6 (a) Ring-closing depolymerization of PHB, (b) reaction between cyclic oligo (3-PHB) and tyrosol [75].

Figure 4.7 Synthesis of oligo((*R*)-3-hydroxybutanoates) (OHB) by fragment coupling [22].